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Abstract: Objective: A short period of warm ischemia during lung allograft implantation is inevitable. We studied the effect of 2 h of warm ischemia before implantation after 18 h of cold preservation on reperfusion edema and pulmonary hemodynamics in a large animal model. Methods: Left lung transplantation was performed in ten weight-matched pigs (25-31 kg). Donor lungs were flushed with 1.5 l cold (1°C) LPD solution and preserved for 20 h. In Group I (n=5) the grafts were preserved for 20 h at 1°C and topically cooled with ice slush during implantation until reperfusion. In Group II (n=5) lungs were stored at 1°C for 18 h followed by 2 h preservation at room temperature (20°C). Topical cooling was not used during implantation. At 1 h after reperfusion the recipient contralateral right pulmonary artery and bronchus were ligated to assess graft function only. Extravascular lung water index (EVLWI), intrathoracic blood volume (ITBV), mean pulmonary artery pressure (PAP) and cardiac output (CO) were assessed during a 4 h observation period. Quantitative myeloperoxidase (MPO) activity and thiobarbituric acid-reactive substance (TBARS) levels as an indicator for lipid peroxidation were determined in allograft tissue samples taken 5 h after reperfusion. Results: In Group II a tendency to improved pulmonary vascular resistance and cardiac output was noted. Surprisingly, lung edema, assessed by EVLWI, did not increase in animals with warm ischemia. Even a tendency to a reduced EVLWI was noted. However, differences between groups did not reach statistical significance. Gas exchange did not differ statistically significant between groups. Conclusion: Our results indicate that a short period of warm ischemia before reperfusion does not lead to increased pulmonary edema. In animals with a short period of warm ischemia before reperfusion, even a tendency to reduced posttransplant lung reperfusion injury was noted. In this model, topical graft cooling during lung implantation did not improve posttransplant graft function

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Effect of a short period of warm ischemia after cold preservation on reperfusion injury in lung allotransplantation[1]

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Abstract

Objective: A short period of warm ischemia during lung allograft implantation is inevitable. We studied the effect of 2 h of warm ischemia before implantation after 18 h of cold preservation on reperfusion edema and pulmonary hemodynamics in a large animal model. **Methods:** Left lung transplantation was performed in ten weight-matched pigs (25–31 kg). Donor lungs were flushed with 1.5 l cold (1°C) LPD solution and preserved for 20 h. In Group I ($n = 5$) the grafts were preserved for 20 h at 1°C and topically cooled with ice slush during implantation until reperfusion. In Group II ($n = 5$) lungs were stored at 1°C for 18 h followed by 2 h preservation at room temperature (20°C). Topical cooling was not used during implantation. At 1 h after reperfusion the recipient contralateral right pulmonary artery and bronchus were ligated to assess graft function only. Extravascular lung water index (EVLWI), intrathoracic blood volume (ITBV), mean pulmonary artery pressure (PAP) and cardiac output (CO) were assessed during a 4 h observation period. Quantitative myeloperoxidase (MPO) activity and thiobarbituric acid-reactive substance (TBARS) levels as an indicator for lipid peroxidation were determined in allograft tissue samples taken 5 h after reperfusion. **Results:** In Group II a tendency to improved pulmonary vascular resistance and cardiac output was noted. Surprisingly, lung edema, assessed by EVLWI, did not increase in animals with warm ischemia. Even a tendency to a reduced EVLWI was noted. However, differences between groups did not reach statistical significance. Gas exchange did not differ statistically significant between groups. **Conclusion:** Our results indicate that a short period of warm ischemia before reperfusion does not lead to increased pulmonary edema. In animals with a short period of warm ischemia before reperfusion, even a tendency to reduced posttransplant lung reperfusion injury was noted. In this model, topical graft cooling during lung implantation did not improve posttransplant graft function.

Keywords: Reperfusion injury; Lung transplantation; Swine; Topical cooling

1. Introduction

Observing the clinical practice of different lung transplant groups, a great difference in the use of topical cooling with ice slush during implantation was noted. In experimental designs, even a cooling jacket [1] was used. And indeed, the importance of rewarming of the organ before reperfusion has not been studied extensively in the field of lung transplantation.

Storage at low temperatures is of course the basic measure in organ preservation. However, reperfusion of the ice cold organ might have detrimental effects. A slow rewarming phase during implantation which allows better activation of protective endothelial mechanisms at the time of reperfusion might be beneficial, as for example synthesis of prostaglandins and nitric oxide production.

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In this study topical cooling during implantation was compared to a slow rewarming phase before reperfusion in a large animal model of reperfusion injury following unilateral lung transplantation.

2. Materials and methods

2.1. Animals and operative procedure

Ten weight-matched pairs of outbred pigs served as donors and recipients. Harvest and left lung transplantation were performed as previously reported [2].

2.2. Donor procedure

Donor animals were intubated with an endotracheal tube and anesthetized with halothane 1.5% (Synmedic, Zürich, Switzerland). Mechanical ventilation was established with 100% oxygen at a tidal volume of 550 ml, a rate of 20 breaths/min and 5 cm H_2O of positive end-expiratory pressure (PEEP). An FiO_2 of 1.0 was maintained throughout the whole procedure. After a median sternotomy, the superior and inferior venae cavae, the ascending aorta, the main pulmonary artery (PA) and the trachea were isolated. Animals were heparinized (400 U/kg), and a curved metal tipped cannula was inserted through a purse string suture in the main PA just distal to the valve. Before administration of the flush solution, 250 mg prostaglandin E_1 (PGE_1) (Prostin VR Pediatric; The Upjohn Company, Kalamazoo, MI) was injected directly into the main PA. Cardiac inflow was occluded by ligation of the superior and inferior venae cavae 20 s after the infusion of PGE_1. The inferior vena cava was cut proximal to the ligation and the tip of the left atrium was excised for decompression of the PA flush. The lungs were perfused immediately, at a pressure of 40 cm H_2O, with 1.5 l LPD solution (Perfadex®, Upjohn/Medisan Pharmaceuticals AB, Uppsala, Sweden). During the flush the lungs were cooled topically by flooding the chest with cold (4°C) saline solution (0.9%). When the flushing was completed, the trachea was clamped at mid-inspiration and the heart–lung block was excised. The harvested organs were stored in LPD solution (1°C) for 20 h before implantation.

2.3. Recipient procedure

Recipient animals were placed in the right lateral decubitus position after intubation with a single lumen endotracheal tube and ventilated with FiO_2 1.0 and 1.5% isoflurane (Abbott, Baar, Switzerland). The left jugular vein and artery were exposed and cannulated for the introduction of a Swan–Ganz catheter and the thermistor-tipped fiberoptic catheter.

A left thoracotomy was performed in the fifth intercostal space. The hemiazygos vein was ligated and divided, and a left pneumonectomy completed after dissection of the hilum. The right PA, the pulmonary arterial branch to the right upper lobe, and the right intermediate bronchus were encircled with 1-0 silk ligatures or umbilical tape. This was in preparation to exclude the right lung from perfusion and ventilation following reperfusion to assess allograft function only. The donor left lung was then isolated from the heart–lung block.

With a partial occlusion clamp in place the left atrium was opened between the superior and inferior pulmonary vein and prepared for anastomosis. The left atrial anastomosis was carried out with a running monofilament suture (Prolene 5-0) using an everting mattress technique. The pulmonary artery anastomosis was completed with a monofilament suture (Prolene 5-0) using a continuous over-and-over technique. A Fogarty venous embolectomy catheter was passed across the field as a bronchial blocker after division of the left mainstem bronchus. The bronchial anastomosis was carried out with a running 4-0 monofilament suture (Prolene) using simple non-telescoping technique. One hour after reperfusion the right main PA, the arterial branch to the upper lobe and the right intermediate bronchus were ligated. The right upper lobe was excluded from ventilation by advancing the tracheal tube to the carina. Subsequently, two chest tubes were inserted into the thoracic cavity and placed on suction. The thoracotomy was closed with umbilical tape and continuous sutures.

2.4. Study groups

In Group I ($n = 5$) donor lungs were harvested as described above and preserved for 20 h at 1°C. During separation and implantation the lungs were cooled topically with ice slush. In Group II ($n = 5$) the lungs were harvested in identical technique and preserved for 18 h at 1°C. Following cold preservation, the lungs were kept at room temperature (20°C) during separation of the left lung. Topical cooling with ice slush was not used and the lung graft was exposed to the body temperature of the recipient during implantation.

All animals received humane care in compliance with the European Convention on Animal Care. The protocol was approved by the local animals study committee.

2.5. Assessment

One hour after reperfusion of the transplanted lung, the right pulmonary arteries and the right main bronchus were ligated to assess allograft function only. During the assessment period anesthesia was maintained with fluothane 1.5%. Systemic arterial, PA, cen-

tral venous and left atrial pressure were recorded continuously. Arterial and mixed venous blood were collected for gas analysis every 60 min.

At the end of the assessment period, 5 h after reperfusion, the animals were sacrificed. Upper lobe allograft samples were submitted to histologic examination and tissue MPO and TBARS assay.

2.6. Extravascular lung water

A fiberoptic catheter (System Cold Z-021, Pulsion, Munich, Germany) is advanced via the external carotid artery into the descending aorta. The indicator bolus consists of two components. Indocyanine green serves as intravascular marker and ice cold 5% glucose as a thermal intra- and extravascular indicator. The bolus is injected via the external jugular artery with a temperature controlled injector. The dilution curves for dye and temperature are recorded simultaneously in the descending aorta with the thermistor tipped fiberoptic catheter. Thoracic intravascular and extravascular fluid volumes are determined based on the measurement of the mean transit times for thermal and dye indicators and of the decay time volumes calculated from the indicator dilution curves as described previously [3]. The lung water computer (System Cold Z-021, Pulsion) determines the mean transit time for the thermal indicator and for the dye indicator and calculates total thermal volume (ITTV), intrathoracic blood volume (ITBV), and extravascular thermal volume (ETV) [4]. ETV, respectively EVLW are calculated as follows: $ETV = ITTV - ITBV$. All measurements were made hourly in triplicate. The mean value was used for analysis.

2.7. Myeloperoxidase assay

Lung samples were frozen immediately and stored at -70°C until assay. Quantitative MPO activity was determined as previously described [5]. Frozen lung tissue (100 mg) was homogenized in 1 ml of 0.5% hexadecyltrimethylammonium bromide (to release myeloperoxidase (MPO) from the primary granules of the PMN), 5 mmol/l EDTA, and 50 mmol/l potassium phosphate buffer (pH 6.2) with a tissue grinder. The homogenate was centrifuged at $10000 \times g$ for 15 min at 4°C. The supernatant was assayed for total soluble protein by the method of Pierce Laboratories [6] and for MPO activity. Enzyme activity was measured spectrophotometrically: 10 mg of 5-fold supernatant was combined with 0.6 ml Hanks' BSA, 0.5 ml of 100 mmol/l potassium phosphate buffer (pH 6.2), 0.1 ml 0.05% H_2O_2, and 0.1 ml of 1.25 mg/ml *o*-dianisidine. Color development was stopped by addition of 1% NaN_3 after five, respectively, 20 min at room temperature. The optical density was measured at 460 nm with a spectrophotometer (Kadas 100, Dr.Lange AG Zürich, Switzerland). Color development from 5 to 20 min was linear. Enzyme activity is expressed as change in optical density unit per milligram of tissue protein per minute (ΔOD/mg per min).

2.8. Thiobarbituric acid reactive substances

Lung samples were frozen immediately and stored at -70°C until assay. TBARS were measured according to the method of Ohkawa et al. [7] in 10% wet weight per volume homogenate. Aliquots (0.2 ml) of this homogenate were added to tubes containing 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with NaOH, and 1.5 ml of 0.8% solution of thiobarbituric acid. The mixture was brought to a volume of 4 ml by the addition of distilled water, heated at 95°C for 60 min, and then cooled with tap water. A volume of 1 ml of distilled water and 5 ml of butanol/pyridine (15:1, v/v) were added (all chemicals by Fluka AG, Switzerland). The solution was centrifuged at 4000 rpm for 10 min. The absorbance of the upper layer was measured at 532 nm with a spectrophotometer (Kadas 100). The TBARS levels were determined by reference to a standard curve of 1,1,3,3-tetramethoxypropane (Sigma), and the results were expressed as picomoles of malondialdehyde per gram of wet lung.

2.9. Statistical analysis

All values are given as the mean $\pm$ standard error of the mean (S.E.M). Analysis for repeated measures was performed by ANOVA (Statistica 4.5 StatSoft 1993) and planned comparison was used. Differences were considered significant at the $P < 0.05$ level.

3. Results

3.1. Characteristics of experimental groups

Donor weight was 28.1 ± 0.9 kg and recipient weight 27.6 ± 2.3 kg in Group I. In Group II the weights were 28.2 ± 1.3 and 27.8 ± 1.3 kg, respectively. There was no statistical difference between groups.

Preservation time was 1217.6 ± 16.5 min in Group I and 1215.0 ± 16.0 min in Group II (difference not significant). Mean warm ischemia in Group II was 148.2 ± 7.3 min. Body temperatures of recipients did not differ in both groups (35.3 ± 0.26 in Group I, 35.2 ± 0.37°C in Group II).

3.2. Gas exchange

P_aO_2 did not differ at baseline (63.9 ± 6.8 kPa in Group I and 66.9 ± 3.1 kPa in Group II). At 1 h after occlusion of the right lung, the P_aO_2 dropped to 50.8 ± 13.1 kPa in Group I, whereas in Group II, a higher P_aO_2 of 57.7 ± 7.4 kPa was measured. This difference was not statistically significant. At 4 h after reperfusion, the difference in P_aO_2 was even higher (Group I: 48.7 ± 12.4 vs. Group II: 61.4 ± 7.8 kPa), but not statistically significant.

3.3. Hemodynamic parameters

Cardiac output was lower at the baseline in Group II (Fig. 1). At 1 h after occlusion of the right lung, CO was 3.06 ± 0.10 l/min in Group I and 3.56 ± 0.15 l/min in Group II ($P = 0.06$). This trend to an improved cardiac output in the group with warm ischemia persisted during the whole observation period, however, no statistically significant difference was noted.

Mean pulmonary artery pressure was the same in both groups at baseline. At 1 h after occlusion, a slightly lower PAP_{mean} in Group II compared to Group I was observed (32.4 ± 1.9 vs. 34.0 ± 1.8 mmHg, $P = 0.62$). At the end of the assessment, the mean PAP was 32.0 ± 3.0 mmHg in Group I and 29.8 ± 1.3 mmHg in Group II ($P = 0.65$).

Pulmonary vascular resistance was higher in Group II at baseline (159.0 ± 27.3 vs. 121.8 ± 16.8 dyne/s per cm^{-5}, $P = 0.45$). At 1 h after occlusion of the contralateral right lung PVR was higher in the group with topical cooling (603.0 ± 44.0 vs. 532.0 ± 48.5 dyne/s per cm^{-5}, $P = 0.41$) but 4 h after occlusion PVR was lower in Group I than in Group II (554.5 ± 109 vs. 570.9 ± 62.1 dyne/s per cm^{-5}, $P = 0.73$). No statistical significant difference in pulmonary vascular resistence was found during the whole observation period.

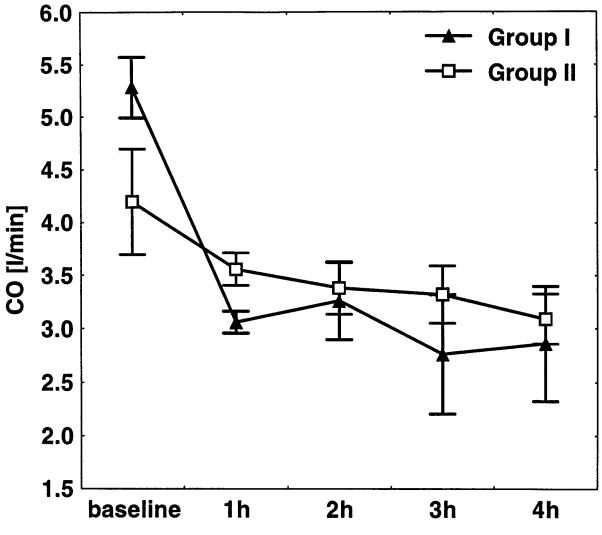

Fig. 1. Cardiac output at baseline and 1, 2, 3 and 4 h after occlusion of the right contralateral lung (mean ± S.E.M.).

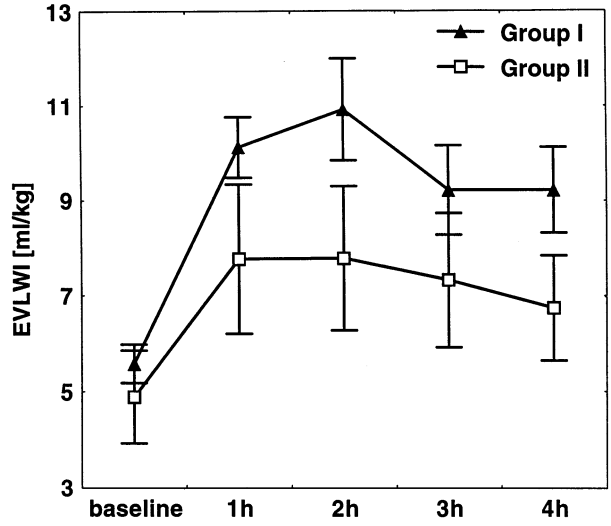

Fig. 2. EVLWI at baseline and 1, 2, 3 and 4 h after occlusion of the right contralateral lung (mean ± S.E.M.).

3.4. Reperfusion edema

After occlusion of the right lung, one hour after reperfusion, only EVLWI of the graft was measured. EVLWI at this time point was lower in Group II (7.77 ± 1.57 ml/kg) compared to Group I (10.12 ± 0.64 ml/kg, $P = 0.25$). This trend to reduced edema (Fig. 2) in the group without topical cooling persisted until four hours after occlusion (6.74 ± 1.1 vs. 9.22 ± 0.90 ml/kg, $P = 0.23$). However, differences were not significant.

Intrathoracic blood volume showed no differences between the groups.

3.5. PMN migration

A tendency to a reduced MPO-activity in Group II (3.25 ± 0.19 ΔOD/mg per min), compared to Group I (4.52 ± 0.64 ΔOD/mg per min), was noted at the end of the observation period ($P < 0.08$) Fig. 3.

3.6. Lipid peroxidation

No difference of TBARS in tissue homogenates between groups was seen 5 h after reperfusion: Group I 12.07 ± 0.86 pmol/kg compared to Group II 12.07 ± 1.18 pmol/g (Fig. 3).

4. Discussion

Various experiments have been conducted to evaluate the optimal preservation conditions in lung transplanta-

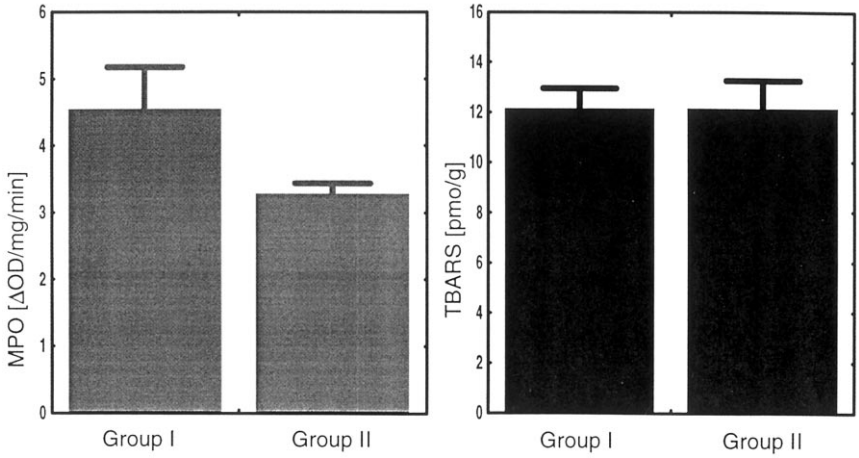

Fig. 3. MPO-Assay and TBARS in graft tissue at the end of the observation period (mean ± S.E.M.).

tion; and the mechanisms of ischemia-reperfusion injury have been studied extensively in the field of thoracic organ transplantation. However, to our knowledge the beneficial effect of topical lung cooling during implantation with subsequent rapid reperfusion of the cold organ on posttransplant lung edema and hemodynamics has not been evaluated.

In the present experiment we compared the effect of topical cooling during implantation to prewarming of the organ for a 2-h period at room temperature prior to reperfusion. Surprisingly we found that prewarming of the lung allograft and avoidance of topical cooling with ice slush did not impair post-transplant graft function. On the contrary, a tendency to improved posttransplant hemodynamics, reduced reperfusion edema, and reduced PMN migration to the allograft was noted. The beneficial effect on pulmonary vascular resistance was most marked in the early phase of reperfusion.

Without focusing directly on the problem of rewarming a number of previously performed studies by other groups support our finding that low temperature of the organ at the time of reperfusion might enhance the development of reperfusion injury and vascular dysfunction. Moriyasu et al. [8] studied the effect of an initial warm (20°C) crystalloid flush before regular reperfusion after a 6-h preservation period in an ex vivo rabbit model. The flush was used to eliminate toxic metabolites from the vascular system. The observed improvement of pulmonary vascular resistance after reperfusion might be as well a consequence of rewarming of the graft before reperfusion. Bhabra et al. demonstrated in a rat lung transplant model that initial low-pressure reperfusion for 10 min reduces posttransplant lung edema [9]. Also in this experiment it is possible that the amelioration of reperfusion injury is at least partially due to a rewarming effect before reperfusion.

Van Raemdonk et al. demonstrated that lung flush is the most effective way to change the temperature of the graft [10]. Reperfusion of the organ with blood at body temperature, therefore, induces a rapid increase of the tissue temperature in the graft which might disturb reorganisation of the cytoskeletal components of the endothelial cells which demonstrate abnormal configuration already during cold preservation [11]. It was shown that lungs are able to maintain aerobic metabolism during storage at 10°C and that this temperature seems to be the ideal storage temperature for lungs [12,13]. Prewarming activates cell metabolism in a similar way which might have a beneficial effect on posttransplant lung injury.

In addition, Ingemansson et al. demonstrated in a series of experiments that cold preservation severely impairs endothelial dependent and endothelial independent vascular relaxation after reperfusion [14] and that cold ischemia and reperfusion independently induce vascular dysfunction. Our results indicate that reperfusion with subsequent rapid rewarming of the lung allograft further increases vascular dysfunction. On the other hand we could not demonstrate that the short period of warm ischemia before reperfusion enhances the production of oxygen free radicals substantially as lipid peroxidation products, assessed by the measurement of tissue-TBARS 5 h after reperfusion, were not increased in the group without topical cooling. We also observed a strong tendency towards a reduction of PMN migration to the allograft which correlates in many models with the severity of posttransplant lung injury. This reduction might be caused by reduced neutrophil sequestration in the lung, due to reduced sheer forces by improved hemodynamics, and decreased PMN and endothelial activation.

In addition, reperfusion injury in this new experimental setting was evaluated by the measurement of extravascular lung water which is an excellent parameter

to assess minor differences in posttransplant lung edema [4]. Clinical studies demonstrated that EVLWI correlates better to the pathological status of the lung than gas exchange and radiological signs of edema [15,16]. A 20-h preservation time was chosen because following this ischemic injury the recipients still tolerate ligation of the right pulmonary artery which allows to assess isolated graft function. If a longer preservation time is used a rapid decrease of allograft gas exchange is noted and the animals die from right heart failure.

In our hands temperature measurement in the allograft bronchus was unreliable, therefore mimicking the clinical situation, the preservation temperature in the ice box, the room temperature and the recipient's body temperature were measured and kept constant in all experiments. Measuring the lung core temperature would have harmed the graft. So we are not able to prove the hypothesis that the 2-h period of warm ischemia led to a remarkable higher core temperature. However, it was not our intention to show the time course of lung parenchyma temperatures with topical cooling versus prewarming but to imitate the clinical situation in lung transplantation and compare topical cooling with the use of no topical cooling.

In conclusion this study in a large animal model of reperfusion injury following unilateral lung transplantation indicates that topical cooling of the allograft with ice slush during separation and implantation seems to be unnecessary. Posttransplant edema and hemodynamics in the group without topical cooling were at least as good in this model than in recipients in which the lung grafts were cooled topically and reperfused at low tissue temperature.

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Appendix A. Conference discussion

Dr G. Petterson (*Copenhagen*, *Denmark*): Very interesting and possibly somewhat surprising results. I had some concern when we did bronchial artery revascularization and allowed the mammary artery to be open during the course of the procedure, early bronchial artery reperfusion could be harmful. I have the impression that that was not the case.

Dr D. van Raemdonck (*Leuven*, *Belguim*): I have a few questions. First, did you measure lung core temperature in the second group while this allograft was rewarming? The second question, during the cold preservation of 20 h did you keep the lung inflated, because it's well known that warm ischemic tolerance is prolonged during inflation and clinically during implantation you cannot keep the lung inflated. Finally, what is your advice for us clinical lung transplant surgeons? Do we need to cool the lung during implantation or not?

Dr U. Stammberger: Firstly, we did not measure temperature in the lung allograft because it is known, for example, by the work of van Raemdonck, that measuring temperature in the bronchus is unreliable since it does not represent tissue temperature, and all other techniques to measure temperature in the lung would harm the allograft tissue. In answer to the second question, the lungs were inflated during storage and were deflated during implantation. To your last question, our clinical practice in Zurich is that we use some amount of ice slush in the thoracic cavity but the lung is not embedded in the ice slush. This experiment has confirmed that we do not have to change this practice since maximal topical cooling seems not to be superior.

Dr N.J. Odom (*Manchester*, *UK*): Interesting results and in a way the opposite to what one would have expected. Do you have any theory as to why the lungs that are allowed to warm up seem to do better? In relation to that, may I ask, was there any dextrose in your preservation solution? It has been shown that lungs stored at 10°C, if there is a metabolic substrate present, perhaps they do better, and might that be a possible explanation? But I don't know if you had dextrose in your preservation solution or not. Where do you go from here? How might you explain this? What do you think might be going on?

Dr U. Stammberger: We use Perfadex as the flush solution and we also have similar hypotheses on the mechanism during rewarming. Maybe the short period of warm ischemia allows the endothelial cells to establish metabolism to a level that let them better cope with reperfusion injury, but in the presented experiment we did not study these mechanisms.

D.R. Metras (*Marseille*, *France*): I want to congratulate Dr Stammberger for an extremely elegant study and very strange results. The only thing which is of concern to me is the temperature of your cold perfusate. It looks like it's 1°C and topical ice slush. It looks extremely cold to me, and I thought that too cold was not that good for the preservation of tissues (this has been shown for the heart). I want to know why you chose so low a temperature?

Dr U. Stammberger: It is well known that the ideal temperature for lung preservation is around 10°C. However, in clinical practice the organ is harvested and stored in a cooling box with a mixture of ice and water. So, we choose the same temperature as in clinical practice. We might have seen other results if we choose 10°C storage temperature.

A. Haverich (*Hannover*, *Germany*): Now, in clinical practice the true consequences of reperfusion injury are usually seen after 12–16 h. Your observation period was only 4 h. Would you expect that with a longer observation period the results might have been different?

Dr U. Stammberger: Extravascular lung water is a very early parameter of reperfusion injury. So, in our opinion, the 5-h period (1 h reperfusion, then occlusion of the contralateral lung, 4 h assessment) is sufficient. Moreover, at the end of this period, extravascular lung water was again decreasing in this model. We cannot predict what the situation might have been after 12 or 16 h. In clinical use ischemic times up to 6 or 7 h are accepted. After this shorter ischemic period the consequences of reperfusion injury might be delayed.